EVALUATION OF ELEVATED DIETARY IRON AND ALUMINUM ON GROWTH AND SURVIVAL OF PACIFIC WHITE SHRIMP *LITOPENAEUS VANNAMEI*

A Thesis

by

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ABSTRACT

The need to identify economical applications for the co-products of algal biofuel has led to the evaluation of these co-products as feedstuffs in aquaculture feeds. However, the flocculation of algae often results in co-products with high levels of iron (Fe) and aluminum (Al). Two experiments were conducted to evaluate the effects of high levels of dietary Fe and Al on *Litopenaeus vannamei*. For the first experiment, a semi-purified basal diet was evaluated using ten diets containing graded levels of either Fe or Al at 200, 500, 1,000, 2,000, or 3,000 mg/kg diet over a 30-day period. In the second experiment, 12 diets with either Fe inclusions of 1,650, 3,260, 4,910, 6,640, 8,290 or 10,044 mg/kg diet or Al inclusions of 670, 1,330, 2,000, 2,702, 3,370, or 4,050 mg/kg diet were evaluated over a 42-day period.

The experiments were conducted with post-larval shrimp in 24-L aquaria using a flow-through system. Dissolved oxygen, temperature, and salinity were monitored daily. Automatic feeders distributed diet 15 times daily based on an estimated average growth curve for the duration of both experiments. Feces, molts and uneaten feed were siphoned daily.

Survival was not affected in either experiment by the dietary treatments. In the first experiment, growth and biomass increased with each level of supplemental Fe and Al to the 1,000 mg/kg inclusion. At 2,000 mg/kg inclusion of either Fe or Al, growth and biomass decreased significantly. In the second experiment, growth and biomass significantly decreased with increasing inclusion of either mineral ($P<0.0001$). In both experiments, body tissues were analyzed to determine mineral retention. In the first

experiment, tail muscle and whole-body Fe and Al tissue levels did not increase. However, hepatopancreactic levels significantly increased with increasing dietary levels of both Fe and Al. In the second experiment, retention in tail muscle and combined head and carapace tissues increased significantly with increasing dietary inclusion levels of both Fe and Al.

Based on data from these experiments, relatively high levels of less than 10,000 mg/kg of Fe and Al in co-products are safe assuming a dietary inclusion level of 10% of the co-product.

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1. INTRODUCTION

In 2011, aquaculture contributed 40% of the world's fish, invertebrate and plant production. Production of food from aquaculture reached nearly 63 million tons, which represents an increase of more than 6% from 2010 to 2011 (FAO, 2013). Aquaculture is a rapidly growing industry that plays an important role in food production worldwide. The production of plants and animals from aquaculture is growing at a rate of 7% a year worldwide (Duarte et al., 2009).

Feed manufacturers have traditionally and currently rely on capture fisheries as a source of fishmeal and fish oil to provide essential dietary protein and lipid in diets for aquatic species. Between 1980 and 2010, the amount of fishmeal used for aquaculture has risen from 10% to 73% of the total available supply, and 71% of fish oil produced today is used in aquaculture (IFFO, 2013). Due to increased knowledge of dietary requirements for aquatic animals, alternative animal and plant sources are being used to replace fish meals and oils in diets. Fishmeal and oil are a valuable source of highly unsaturated fatty acids and polyunsaturated fatty acids (HUFAs and PUFAs) critical to fish development (Naylor et al., 2009). However, it is understood that these fisheries products are a finite resource high in demand. Therefore, alternative sources of protein and lipid for use in aquaculture diets must be identified for both sustainability and economic reasons (Patnaik et al., 2006).

The growing world-wide interest in alternative energy has increased the production of micro- and macro-algae for use as biofuels. Algae produced primarily for

biofuel undergoes an extraction process leaving behind a potentially valuable co-product. This lipid-extracted algae (LEA) has created new opportunities for animal feed industries (Singh et al., 2011). Some species of algae are rich in oil that contains HUFAs, as well as protein (Ju et al., 2009). The oil-rich whole algae and LEA also has the potential to be used directly as a feed ingredient, partially or completely substituting fish oil, fish meal, and/or plant meals (Patnaik et al., 2006). This is a high research priority since it would reduce the pressure placed on wild fish populations and capture fisheries for providing fishmeal and fish oil.

Analysis of LEA has demonstrated that much of the nutritional quality remains intact after the oil has been extracted for biofuel, and research is being done to evaluate the value of these co-products as feed ingredients for aquaculture (Ju et al., 2012). The amount of oil remaining in LEA ranges from 6-9%, making it an ideal lipid source for shrimp feeds. The use of co-products from algae production for biofuels as feed ingredients for fish and shrimp diets would add value to algae production by providing additional potential revenue. Also, LEA co-product has the potential to reduce the greatest variable cost in aquaculture, the cost of feed (FAO, 2006), while potentially increasing the nutritional quality of aquaculture feeds.

Therefore, three central reasons exist to consider LEA as a feed ingredient in shrimp aquaculture. Firstly, the high nutritional quality of the extracted algae co-product presents the potential to increase shrimp growth rate. Secondly, the cost of the algae co-product could be an economical solution to the increasing price of fishmeal and fish oil. Partial or complete substitution of LEA for fishmeal should decrease the cost of

commercial shrimp feed. Finally, the estimated commercial value of LEA at $600-1000 per ton increases the commercial value of producing algae for biofuels.

One step in the processing of microalgae for biofuel is flocculation, in which suspended cells of algae are coalesced into loosely packed conglomerates prior to harvest (Chen et al., 2011). Commonly used flocculation agents are ferric chloride and ferric sulfate (Grima et al., 2003), as well as aluminum sulfate (Rwehumbiza et al., 2012). After flocculation, the metal content of the algae is elevated. These elevated levels of iron (Fe) and aluminum (Al) in LEA co-product pose a potential concern to feed manufacturers and animal producers (Frías-Espericueta et al., 2009). During the initial evaluation of algae co-product, a complete mineral analysis revealed elevated levels of aluminum and iron. In order to consider LEA co-product as a feedstuff for aquaculture, the potential toxicity of these metals must be evaluated. Additionally, because the shrimp produced via aquaculture are intended for human consumption, the retention of metals in the muscle tissue must be determined. However, no published peer-reviewed papers concerning the retention of Fe or Al in shrimp tissues were identified. Furthermore, there are no published peer-reviewed papers concerning the tolerance of penaid shrimp to elevated levels of dietary Fe or Al.

Thus, the objectives of this research were to (1.) determine if the levels of Fe and Al contained in LEA co-product are detrimental to shrimp growth or survival, and (2.) to determine to what levels Fe and Al are retained in shrimp tissues.

2. METHODS

2.1 Low Inclusion Experiment

2.1.1 Shrimp

Specific-pathogen-free Litopenaeus vannamei were obtained as postlarvae (PLs) from the Oceanic Shrimp Improvement Systems LLC, Plantation Key, Florida, and stocked into laboratory tanks at a water depth of 20 cm. PLs were fed live brine shrimp (Artemia sp. nauplii) and a simulated commercial diet twice and 12 times daily, respectively. PLs were reared to the desired initial stocking size using the standard methods practiced at Texas AgriLife Research Shrimp Mariculture Project in Port Aransas, TX and were allowed to acclimate to laboratory conditions and achieve proper weight for the experiment.

2.1.2 Feed Preparation

Feeds were prepared in 3-kg batches. For each diet, dry ingredients and oils were mixed in a V-mixer for one hour and then transferred to a food mixer (Model A-200, Hobart Corporation, Troy, OH). In a separate bowl, alginate and sodium metaphosphate were added to deionized water and mixed using a hand mixer (Sunbeam Products Inc., Milford, MA) for approximately 45 seconds. The alginate mix was then added to the dry ingredients and mixed at room temperature (~24ºC) for an additional minute to achieve a mash consistency appropriate for extrusion. Extrusion was accomplished at room temperature using a meat chopper attachment (Model A-800, Hobart Corporation, Troy,

OH) fitted with a 3-mm die. Moist feed strands were dried on wire racks in a forced air oven at 35 ^{o}C to a moisture content of 8-10%. After a 24-h drying period, feed was milled and sifted into appropriate size for shrimp consumption, bagged, and stored at 4 ^{o}C until used.

2.1.3 Feed Ingredients and Nutrients

Ten experimental diets were prepared in addition to a basal control diet. Table 1 shows the ingredient composition of basal diet. This basal diet is a semi-purified diet comparable to a commercial feed used in clear water systems. Either Fe or Al was added into the experimental diets on a mg/kg basis at the following levels: 200, 500, 1000, 2000, 3000. In experimental diets, diatomaceous earth was replaced with either aluminum sulfate (Al2(SO4)3*18H2O) or iron sulfate (FeSO4*7H2O), with the exception of the 2000 and 3000 mg/kg of inclusion of Al, which also replaced minimal amounts of wheat starch as shown in Table 2. This allowed all nutrients to be relatively constant except for the Fe and Al levels, as well as the carbohydrate and energy levels in the 2000 and 3000 mg/kg inclusions of the Al diets.

Table 1. Basal diet ingredient levels (as-fed basis) for both experiments.

Basal Diet Ingredient Levels in %			
Ingredient	**%**	**Ingredient**	**%**
Alginate	2.00	Oil, Soybean	0.60
Ca Carbonate	2.50	Phospholipid, 97%	4.00
Cellulose	3.20	CaHPO4	4.20
Cholesterol	0.20	NaHexaMetaPO4	1.00
Diatomaceous Earth	3.80	PM Min/Vit I*	0.25
Fishmeal, Menhaden	8.00	PM Min/Vit II*	0.21
KCl	1.90	Soybean-90%	5.70
MgO, feed grade	1.60	Squid, Muscle	30.00
NaCl	0.70	Vit C, Stable35%	0.04
Fish Oil, Menhaden	0.60	Wheat Starch	29.50

*The composition of the Mineral/Vitamin Premixes I & II are given in Appendices A and B, respectively.

Table 2. Ingredient levels for diets supplemented (as-fed basis) with Fe and Al in the low inclusion experiment. All other ingredients were constant in all diets.

	Supplemental Fe Levels in mg/kg & (%)					
Diet Ingredient Level	**Base Diet**	**200 (0.02)**	**500 (0.05)**	**1000 (0.10)**	**2000 (0.20)**	**3000 (0.30)**
Wheat Starch	29.5	29.5	29.5	29.5	29.5	29.5
Diatomaceous Earth	3.8	3.7	3.55	3.31	2.81	2.32
Fe sulfate	0	0.1	0.25	0.49	0.99	1.48
	Supplemental Al Levels in mg/kg & (%)					
Diet Ingredient Level	**Base Diet**	**200 (0.02)**	**500 (0.05)**	**1000 (0.10)**	**2000 (0.20)**	**3000 (0.30)**
Wheat Starch	29.5	29.5	29.5	29.5	29.13	28.3
Diatomaceous Earth	2.97	2.16	1.33	0.46	0	0
Al Sulfate	0.00	0.25	0.62	1.24	2.47	3.71

The base and experimental diets were analyzed by Midwest Laboratories (Omaha, NE) using standard wet chemistry methods (AOAC) to ensure the accuracy of formulated values. Additionally, all diets were analyzed for moisture, crude protein, total ash, crude fat, calcium, and phosphorus. All determined values were similar to formulated levels. Determined nutrient levels for the basal and experimental diets with supplemental levels of Fe and Al are given in **Tables 3 and 4**, respectively.

Table 3. Determined nutrient levels (dry-matter basis) for all diets with supplemented Fe in the low inclusion experiment. Values in % except for iron, manganese, copper and zinc which are in mg/kg.

	Supplemental Iron Levels in mg/kg & (%)					
Nutrient	**Base Diet**	**200 (0.02)**	**500 (0.05)**	**1000 (0.10)**	**2000 (0.20)**	**3000 (0.30)**
Crude protein	39.5	39.5	40.3	39.7	38.6	38
Crude fat	7.6	6.88	7.36	6.88	7.6	7.68
Ash	19.4	19.1	18.8	19	18.7	18.6
Phosphorus	2.16	2.14	2.26	2.28	2.25	2.29
Potassium	1.5	1.62	1.66	1.68	1.66	1.69
Magnesium	1.2	1.23	1.25	1.29	1.27	1.28
Calcium	3.18	3.12	3.26	3.28	3.29	3.39
Sodium	1.09	1.27	1.35	1.31	1.33	1.33
Iron	568	616	1022	1537	2667	3890
Manganese	26	24	27	26	27	30
Copper	49	48	54	50	51	50
Zinc	180	189	201	195	191	196

Table 4. Determined nutrient levels (dry-matter basis) for all diets with supplemented Al in the low inclusion experiment. Values in % except for iron, manganese, copper, zinc and aluminum which are in mg/kg.

	Supplemental Aluminum Levels in mg/kg & (%)					
Nutrient	**Base Diet**	**200 (0.02)**	**500 (0.05)**	**1000 (0.10)**	**2000 (0.20)**	**3000 (0.30)**
Crude protein	39.5	40.2	38.7	39.4	40.2	40.2
Crude fat	7.6	6.42	6.81	9.62	7.5	7.38
Ash	19.4	19.2	18.6	18.4	17.8	16.9
Phosphorus	2.16	2.05	2.05	2.01	2.03	2.15
Potassium	1.5	1.49	1.5	1.46	1.48	1.58
Magnesium	1.2	1.17	1.17	1.16	1.15	1.22
Calcium	3.18	3.03	3.04	2.92	2.91	3.15
Sodium	1.09	1.16	1.16	1.16	1.18	1.25
Iron	568	394	360	310	237	134
Manganese	26	22	22	21	21	22
Copper	49	46	45	49	48	51
Zinc	180	174	175	174	180	188
Aluminum	298	513	821	1280	2257	3323

These data confirmed that all diets contained very similar levels of the analyzed nutrients. Also, the determined values of Fe and Al were very similar to the calculated values.

2.1.4 Protocol

Shrimp were randomly selected and weighed on a per-tank basis before being stocked into 100 covered tanks with water depths of 0.24-m and bottom areas of 0.1-m^2. Air stones to each tank provided the aeration required to keep dissolved oxygen above 5-mg/L^{-1}. Water sourced from the Corpus Christi ship channel was incorporated into a

semi closed (10% new water daily) 43,000-L indoor recirculating system and filtered through 100-μ cartridge filters and UV filters at a recirculation rate of 0.76-liters per minute or 3470% per day. Salinity was allowed to vary with the salinity of the incoming seawater and varied from 35 to 37-ppt. The temperature was controlled at 30±1°C in the recirculating system. Fluorescent bulbs provided lighting on a 12-hour dark and 12-hour light cycle.

To monitor the retention of Fe and Al in the recirculating system, water samples were taken from the sump, the basal diet tanks, as well as the tanks being fed the 200 and 3000-mg/kg inclusion levels of either mineral.

Tanks were siphoned daily to remove excess feed, feces, and molts. Hydrological parameters (dissolved oxygen, temperature, and salinity) were monitored daily using a hydrology meter (YSI 85) and water quality (total ammonia nitrogen, nitrates, nitrites, and pH) were tested weekly using a HACH 2100 spectrophotometer.

For the low inclusion experiment, shrimp were stocked at a density of seven animals per tank with an initial weight of 0.43±0.05 g per shrimp. The initial stocking weight by tank, showing the very similar initial shrimp size for all tanks, is given in **Table 5.** Each level of dietary Fe or Al was assigned to six replicate tanks, while the basal diet was assigned to ten.

Table 5. Stocking weights (in grams) by tank in the low inclusion experiment. Each value represents the total weight of seven shrimp.

Stocking Weights by Tank									
Tank	**(g)**	**Tank**	**(g)**	**Tank**	**(g)**	**Tank**	**(g)**	**Tank**	**(g)**
1	4.52	**21**	4.74	**41**	4.81	**61**	4.81	**81**	4.91
2	4.04	**22**	4.28	**42**	4.61	**62**	4.54	**82**	4.74
3	4.35	**23**	4.56	**43**	4.25	**63**	4.04	**83**	4.65
4	4.78	**24**	4.35	**44**	4.23	**64**	4.39	**84**	5.00
5	4.63	**25**	4.32	**45**	4.30	**65**	4.41	**85**	4.89
6	4.75	**26**	5.00	**46**	4.92	**66**	4.48	**86**	4.87
7	4.05	**27**	4.28	**47**	4.25	**67**	4.57	**87**	4.81
8	4.83	**28**	4.06	**48**	4.80	**68**	4.79	**88**	4.49
9	4.28	**29**	4.05	**49**	4.16	**69**	4.38	**89**	4.37
10	4.48	**30**	4.72	**50**	4.62	**70**	4.81	**90**	4.47
11	4.12	**31**	4.34	**51**	4.05	**71**	4.26	**91**	4.70
12	4.78	**32**	4.28	**52**	4.49	**72**	4.62	**92**	4.57
13	4.09	**33**	4.11	**53**	4.26	**73**	4.87	**93**	4.37
14	4.10	**34**	4.76	**54**	4.05	**74**	4.49	**94**	4.70
15	4.40	**35**	4.18	**55**	4.83	**75**	5.00	**95**	4.36
16	4.32	**36**	4.93	**56**	4.62	**76**	4.63	**96**	4.66
17	4.18	**37**	4.66	**57**	4.30	**77**	4.45	**97**	4.17
18	4.93	**38**	4.36	**58**	4.63	**78**	4.31	**98**	4.32
19	4.48	**39**	4.53	**59**	4.72	**79**	4.71	**99**	4.69
20	4.64	**40**	4.70	**60**	4.97	**80**	4.19	**100**	4.18

Food was administered 15 times daily through the use of automatic feeders. Since feed was always observed in each tank prior to siphoning each day, shrimp were fed above satiation according to an excess feed curve and a relatively high FCR of 2.0. Table 6 shows the amount of feed presented per day to each tank as well as the size of feed presented.

Table 6. Amount and size of feed proffered per day in the low inclusion experiment.

Feed Curve							
Day	**date**	**feed (g)**	**feed size**	**day**	**date**	**feed (g)**	**feed size**
1	11/15/11	1.24	18/14	**15**	11/30/11	4.47	12/10
2	11/16/11	1.38	14/12	**16**	12/01/11	4.64	12/10
3	11/17/11	1.52	14/12	**17**	12/02/11	4.80	12/10
4	11/18/11	1.66	14/12	**18**	12/03/11	4.95	12/10
5	11/19/11	1.80	14/12	**19**	12/04/11	5.09	12/10
6	11/20/11	1.75	14/12	**20**	12/05/11	5.22	12/10
7	11/21/11	2.46	14/12	**21**	12/06/11	5.34	12/10
8	11/22/11	2.72	14/12	**22**	12/07/11	5.46	12/10
9	11/23/11	2.98	14/12	**23**	12/08/11	5.56	12/10
10	11/24/11	3.22	14/12	**24**	12/09/11	5.65	12/10
11	11/25/11	3.45	14/12	**25**	12/10/11	6.00	12/10
12	11/26/11	3.68	14/12	**26**	12/11/11	6.30	12/10
13	11/27/11	3.89	14/12	**27**	12/12/11	6.30	12/10
14	11/28/11	4.09	14/12	**28**	12/13/11	6.30	12/10
15	11/29/11	4.28	12/10	**29**	12/14/11	5.94	12/10
				30	12/15/11	5.97	12/10

Amount fed per day is the product of determined growth of shrimp with starting weight of 0.43±0.05 g per shrimp (Appendix C) and an FCR of 2.0

2.1.5 Data Collection and Tissue Analysis

Shrimp were fasted for a 24-h period before terminating the experiment, and were collected and weighed on a per-tank basis. Shrimp were bagged and frozen by tank assignment. Mineral analysis was performed on whole body, tail muscle, and hepatopancreas samples. Tissue samples from the basal treatment as well as the 200 mg/kg and 3000 mg/kg inclusion of Fe and Al were analyzed. Of the six test replicates for either diet, three tanks were selected for whole-body analysis and three were selected for hepatopancreas and tail muscle analysis.

Data were statistically compared using SAS (version 9.3, 2012-2013) by one-way ANOVA. Treatment means were separated by the Student-Newman-Keuls test ($P<0.05$). Tissue samples were statistically compared using simple regression.

2.2 High Inclusion Experiment

2.2.1 Shrimp

The source and the method of rearing the PLs to the desired initial stocking size were the same as in the low inclusion experiment.

2.2.2 Feed Preparation

Feed preparation methods were the same as used for the low inclusion experiment.

2.2.3 Feed Ingredients and Nutrients

Twelve experimental diets were prepared in addition to a basal control (**Table 1**). Increasing concentrations of Fe were included on a mg/kg basis at the following levels: 1667, 3292, 4962, 6709, 8376, 10044. Al was included on a mg/kg basis at the following levels: 672, 1327, 2000, 2705, 3370, 4048. In experimental diets, diatomaceous earth was replaced with either aluminum sulfate ($Al_2(SO_4)^3*18H_2O$) or iron sulfate ($FeSO_4*7H_2O$), with the exception of the 8376 and 10044 mg/kg inclusions of Fe and the 3370 and 4048 mg/kg inclusions of Al, which also replaced minimal amounts of wheat starch (**Table 7)**.This allowed all nutrients to be relatively constant except for the Fe and Al levels, as well as the carbohydrate and energy levels in the 8376 and 10044 mg/kg inclusions of Fe and the 3370 and 4048 mg/kg inclusions of Al.

Table 7. Ingredient levels (as-fed basis) with supplemented Fe and Al in the high inclusion experiment. All other ingredients were constant in all diets.

	Supplemental Fe Levels in mg/kg & (%)					
Diet Ingredient Level	**1650 (0.17)**	**3260 (0.33)**	**4910 (0.49)**	**6640 (0.66)**	**8290 (0.83)**	**10044 (1.00)**
Wheat Starch	29.5	29.5	29.5	29.5	29.13	28.3
Diatomaceous Earth	2.97	2.16	1.33	0.46	0	0
Fe sulfate	0.83	1.64	2.47	3.34	4.17	5
	Supplemental Al Levels in mg/kg & (%)					
Diet Ingredient Level	**670 (0.07)**	**1330 (0.13)**	**2000 (0.20)**	**2702 (0.27)**	**3370 (0.34)**	**4050 (0.41)**
Wheat Starch	29.5	29.5	29.5	29.5	29.13	28.3
Diatomaceous Earth	2.97	2.16	1.33	0.46	0	0
Al Sulfate	0.83	1.64	2.47	3.34	4.17	5

Diets with supplemented minerals were analyzed by Midwest Laboratories in Omaha, NE using standard wet chemistry methods (AOAC) to ensure the accuracy of formulated values. To confirm consistency between treatments, all diets were also analyzed for moisture, crude protein, total ash, crude fat, calcium, and phosphorus. All values were similar with the exception of Fe and Al, and the determined values of Fe and Al were within 12% of the formulated values. Determined nutrient levels for the basal and experimental diets with supplemental levels of Fe and Al are given in **Tables 8 and 9**, respectively.

Table 8. Nutrient levels (dry-matter basis) for diets with supplemented Fe in the high inclusion experiment. Values in % except for iron, manganese, copper and zinc which are in mg/kg.

	Supplemental Iron Levels in mg/kg & (%)					
Nutrient	**1650 (0.17)**	**3260 (0.33)**	**4910 (0.49)**	**6640 (0.66)**	**8290 (0.83)**	**10044 (1.00)**
Crude protein	38.6	38.7	38	38.8	39.7	39.8
Crude fat	8.64	8.48	8.18	8.7	8.81	7.76
Ash	19	18.9	18.8	18	18	18.4
Phosphorus	2.2	2.21	2.19	2.21	2.11	2.23
Potassium	1.55	1.55	1.53	1.59	1.52	1.57
Magnesium	1.19	1.19	1.19	1.22	1.18	1.24
Calcium	3.23	3.2	3.17	3.16	3.15	3.28
Sodium	1.1	1.09	1.07	1.12	1.08	1.11
Iron	2313	4122	5838	8038	9302	11165
Manganese	25	25	24	24	25	25
Copper	42	43	42	48	39	38
Zinc	173	170	172	177	177	180

Table 9. Nutrient levels (dry-matter basis) for diets with supplemented Al in the high inclusion experiment. Values in % except for iron, manganese, copper, zinc and aluminum which are in mg/kg.

	Supplemental Aluminum Levels in mg/kg & (%)					
Nutrient	**670 (0.07)**	**1330 (0.13)**	**2000 (0.20)**	**2702 (0.27)**	**3370 (0.34)**	**4050 (0.41)**
Crude protein	37.8	38.5	38.4	39.3	38.2	39.7
Crude fat	8.06	7.07	8.11	8.11	7.4	7.99
Ash	18.8	18.5	18.1	17.8	17.5	18
Phosphorus	2.17	2.19	2.21	2.19	2.17	2.18
Potassium	1.53	1.55	1.58	1.55	1.56	1.58
Magnesium	1.2	1.2	1.23	1.21	1.2	1.21
Calcium	3.19	3.23	3.27	3.21	3.2	3.23
Sodium	1.09	1.11	1.12	1.1	1.11	1.1
Iron	452	556	390	481	311	301
Manganese	25	27	25	25	23	24
Copper	45	46	50	44	53	42
Zinc	175	179	187	184	188	184
Aluminum	1073	1855	2691	3431	4130	5009

2.2.4 Protocol

Experimental protocols were the same as used for the low inclusion experiment, with the following exceptions:

For the high inclusion experiment, shrimp were stocked at a density of five animals per tank with a starting weight of 0.90±0.05 g per shrimp. The initial stocking weight by tank, showing the very similar initial shrimp size for all tanks, is given in **Table 10.** Each test diet and the basal control was assigned to eight replicate tanks, while the 670 mg/kg Al and 1650 mg/kg Fe diets were assigned to six replicate tanks.

Table 10. Stocking weights (in grams) by tank in the high inclusion experiment. Each value represents the total weight of five shrimp.

Stocking Weights by Tank									
Tank	**(g)**	**Tank**	**(g)**	**Tank**	**(g)**	**Tank**	**(g)**	**Tank**	**(g)**
1	4.52	**21**	4.74	**41**	4.81	**61**	4.81	**81**	4.91
2	4.04	**22**	4.28	**42**	4.61	**62**	4.54	**82**	4.74
3	4.35	**23**	4.56	**43**	4.25	**63**	4.04	**83**	4.65
4	4.78	**24**	4.35	**44**	4.23	**64**	4.39	**84**	5.00
5	4.63	**25**	4.32	**45**	4.30	**65**	4.41	**85**	4.89
6	4.75	**26**	5.00	**46**	4.92	**66**	4.48	**86**	4.87
7	4.05	**27**	4.28	**47**	4.25	**67**	4.57	**87**	4.81
8	4.83	**28**	4.06	**48**	4.80	**68**	4.79	**88**	4.49
9	4.28	**29**	4.05	**49**	4.16	**69**	4.38	**89**	4.37
10	4.48	**30**	4.72	**50**	4.62	**70**	4.81	**90**	4.47
11	4.12	**31**	4.34	**51**	4.05	**71**	4.26	**91**	4.7
12	4.78	**32**	4.28	**52**	4.49	**72**	4.62	**92**	4.57
13	4.09	**33**	4.11	**53**	4.26	**73**	4.87	**93**	4.37
14	4.1	**34**	4.76	**54**	4.05	**74**	4.49	**94**	4.7
15	4.4	**35**	4.18	**55**	4.83	**75**	5.00	**95**	4.36
16	4.32	**36**	4.93	**56**	4.62	**76**	4.63	**96**	4.66
17	4.18	**37**	4.66	**57**	4.3	**77**	4.45	**97**	4.17
18	4.93	**38**	4.36	**58**	4.63	**78**	4.31	**98**	4.32
19	4.48	**39**	4.53	**59**	4.72	**79**	4.71	**99**	4.69
20	4.64	**40**	4.70	**60**	4.97	**80**	4.19	**100**	4.18

Food was administered 15 times daily through the use of automatic feeders. Since feed was always observed in each tank prior to siphoning each day, shrimp were fed above satiation according to an excess feed curve and a relatively high FCR of 2.0. **Table 11** shows the amount of feed presented per day to each tank, as well as the size of feed presented.

Table 11. Amount and size of feed proffered per day in the high inclusion experiment.

Feed Curve							
Day	**date**	**feed (g)**	**feed size**	**day**	**date**	**feed (g)**	**feed size**
1	6/5/2012	**1.76**	14/12	**22**	6/26/2012	**4.18**	12/10
2	6/6/2012	**1.95**	14/12	**23**	6/27/2012	**4.22**	12/10
3	6/7/2012	**2.13**	14/12	**24**	6/28/2012	**4.25**	12/10
4	6/8/2012	**2.30**	14/12	**25**	6/29/2012	**4.27**	12/10
5	6/9/2012	**2.47**	14/12	**26**	6/30/2012	**4.28**	12/10
6	6/10/2012	**2.63**	14/12	**27**	7/1/2012	**4.29**	12/10
7	6/11/2012	**2.78**	14/12	**28**	7/2/2012	**4.28**	12/10
8	6/12/2012	**2.93**	14/12	**29**	7/3/2012	**4.27**	12/10
9	6/13/2012	**3.06**	12/10	**30**	7/4/2012	**4.26**	12/10
10	6/14/2012	**3.19**	12/10	**31**	7/5/2012	**4.24**	12/10
11	6/15/2012	**3.32**	12/10	**32**	7/6/2012	**4.20**	12/10
12	6/16/2012	**3.43**	12/10	**33**	7/7/2012	**4.17**	12/10
13	6/17/2012	**3.54**	12/10	**34**	7/8/2012	**4.12**	12/10
14	6/18/2012	**3.64**	12/10	**35**	7/9/2012	**4.07**	12/10
15	6/19/2012	**3.73**	12/10	**36**	7/10/2012	**4.01**	12/10
16	6/20/2012	**3.82**	12/10	**37**	7/11/2012	**3.94**	12/10
17	6/21/2012	**3.90**	12/10	**38**	7/12/2012	**3.87**	12/10
18	6/22/2012	**3.97**	12/10	**39**	7/13/2012	**3.79**	12/10
19	6/23/2012	**4.03**	12/10	**40**	7/14/2012	**3.79**	12/10
20	6/24/2012	**4.09**	12/10	**41**	7/15/2012	**3.79**	12/10
21	6/25/2012	**4.14**	12/10	**42**	7/16/2012	**3.79**	12/10

Amount fed per day is the product of determined growth of shrimp with a starting weight of 0.90±0.05 g per shrimp (Appendix D) and an FCR of 2.0.

2.2.5 Data Collection and Tissue Analysis

Data collection and tissue analysis procedures were the same as used for the low inclusion experiment, with the following exceptions:

Mineral analysis was performed on tail muscle and combined head and carapace samples. Tissue samples from the basal treatment, as well as the 670 mg/kg and 4050 mg/kg inclusion of Al and the 1650 mg/kg and 10044 mg/kg Fe treatments were analyzed. All tanks within a treatment were examined for both tail muscle and combined head and carapace.

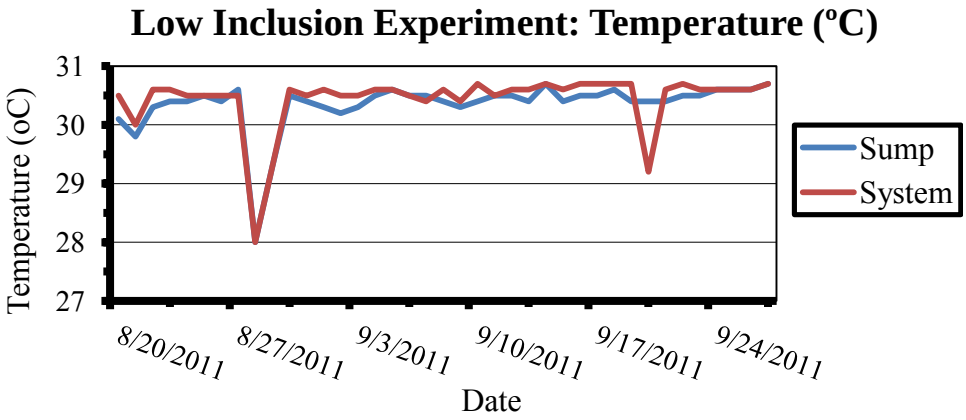
3. RESULTS

3.1 Parameters and Water Quality

3.1.1 Low Inclusion Experiment

As shown in **Figure 1**, temperature within the system remained relatively consistent between 30.0 and 30.5°C, aside from two separate days when the temperature decreased to 28°C and 29°C, respectively.

Figure 1. Temperature data from sump and experimental culture system during the low inclusion experiment.



According to **Figure 2**, the average dissolved oxygen during the low inclusion experiment was 5.5 ppt and ranged between 4.5 and 6.5 ppt, well within the tolerance range of *L. vannamei*.

Figure 2. Dissolved oxygen data from sump and experimental culture system during the low inclusion experiment.

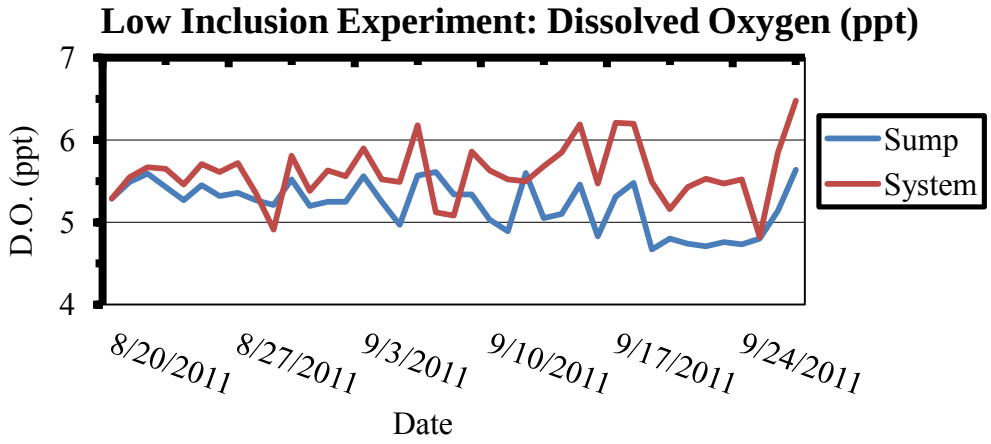
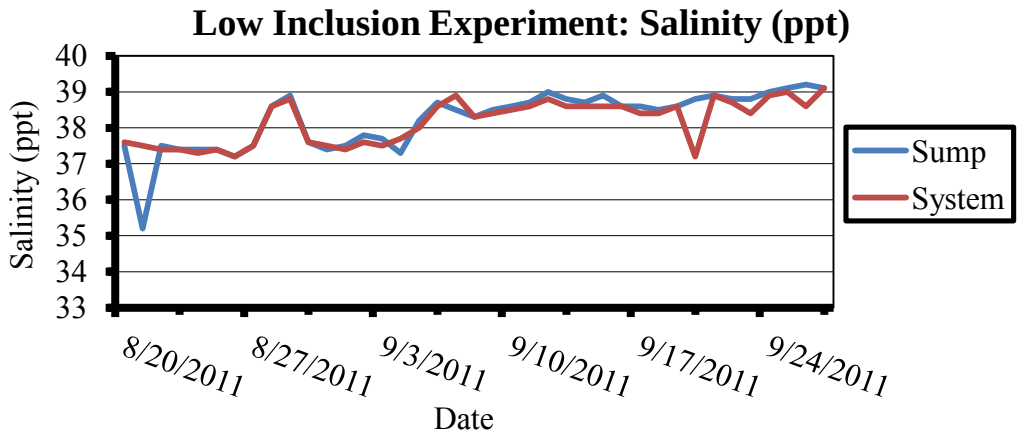


Figure 3 shows the daily measurement of salinity for the low inclusion experiment, between 35 and 39 ppt, well within the tolerance range of *L. vannamei*.

Figure 3. Salinity data from sump and experimental culture system during the low inclusion experiment.



As noted in **Table 12**, water quality parameters were exceptional. TAN, nitrite, and nitrate values were all well below toxic levels for shrimp, and pH values were within acceptable ranges.

Table 12. Total ammonia nitrogen (mg/kg), nitrite (mg/kg), nitrate (mg/kg), and pH of experimental culture system and sump in the low inclusion experiment.

	Sump				System			
Date	**TAN**	**Nitrite**	**Nitrate**	**pH**	**TAN**	**Nitrite**	**Nitrate**	**pH**
8/25/2011	0.06	0.025	1.62	7.76	0.11	0.152	0.61	8.12
9/8/2011	0.07	0.035	1.52	7.50	0.08	0.038	0.88	7.88
9/14/2011	0.06	0.035	0.05	7.50	0.10	0.052	1.18	7.82
9/21/2011	0.09	0.044	2.41	7.49	0.09	0.050	1.52	7.81

Water samples collected from the low and high inclusions of Fe and Al were evaluated for the retention of mineral in the system. No meaningful changes occurred. **Table 13** shows the amount of Fe and Al present in system water.

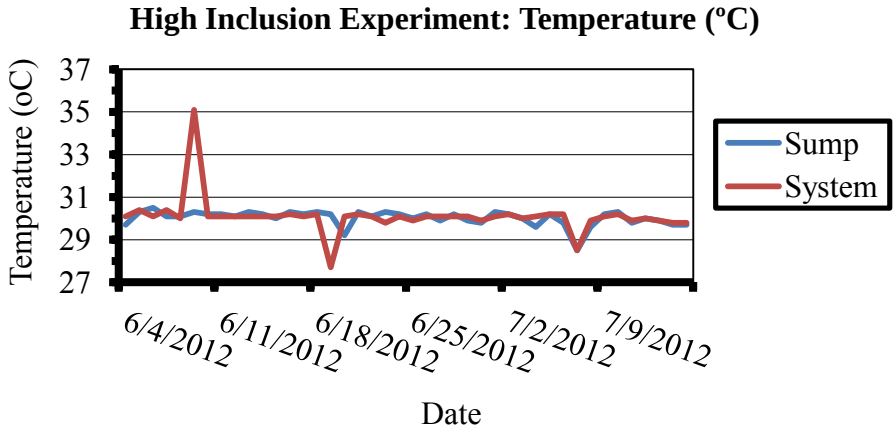
Table 13. Amount of Fe or Al in experimental culture system and sump water in the low inclusion experiment. Values in mg/kg.

	Supplemental Iron Levels in mg/kg & (%)			
Mineral	**Sump (0)**	**Basal (0)**	**200 (0.02)**	**3000 (0.30)**
Iron	0.00	0.00	0.00	0.00
Aluminum	0.03	0.01	0.00	0.01
	Supplemental Aluminum Levels in mg/kg & (%)			
Mineral	**Sump (0)**	**Basal (0)**	**200 (0.02)**	**3000 (0.30)**
Iron	0.00	0.00	0.00	0.00
Aluminum	0.03	0.01	0.01	0.02

3.1.2 High Inclusion Experiment

As shown in **Figure 4**, temperature within the system remained relatively consistent between 30.0 and 30.5°C, with the exception of one day when the temperature spiked to 35°C and two days when it decreased to 28°C and 29°C respectively.

Figure 4. Temperature data from sump and experimental culture system during the high inclusion experiment.



According to **Figure 5**, the average dissolved oxygen during the low inclusion experiment was 5.0 ppt and ranged between 4.5 and 6.5 ppt, well within the tolerance range of *L. vannamei*.

Figure 5. Dissolved oxygen data from sump and experimental culture system during the high inclusion experiment.

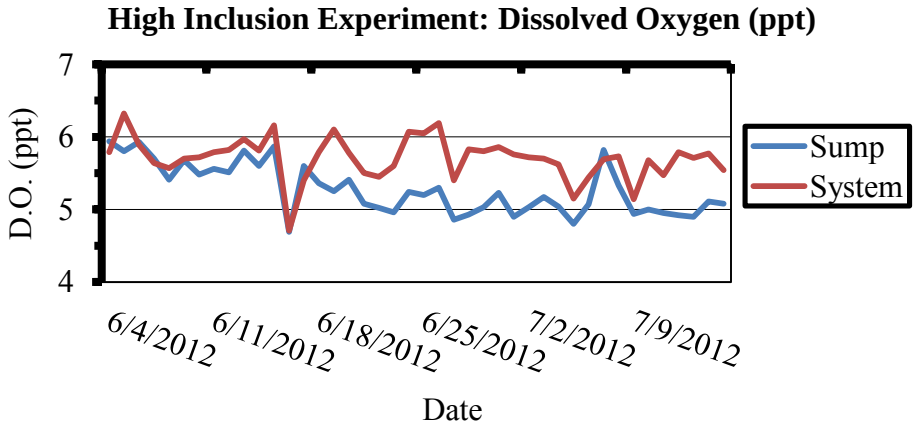
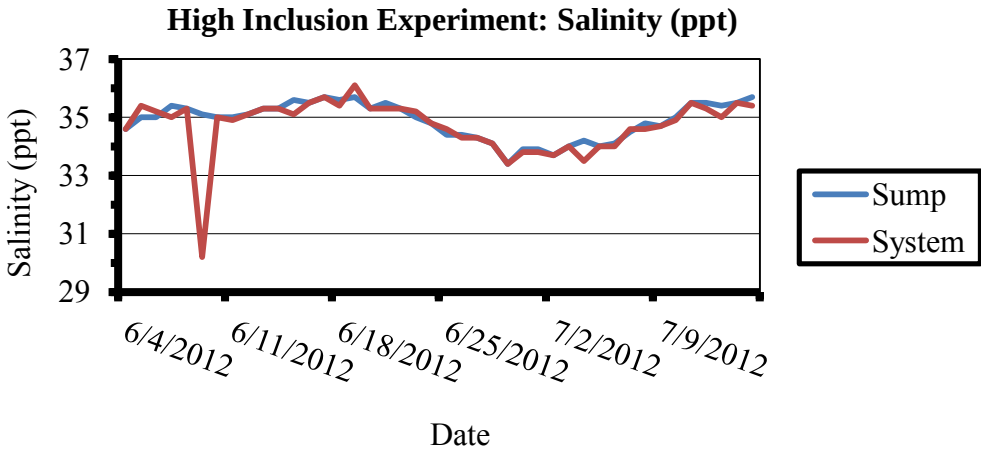


Figure 6 shows the daily measurement of salinity for the low inclusion experiment varied between 33 and 36 ppt, with the exception of one day where the salinity decreased to 30 ppt. These values are well within the tolerance range of *L. vannamei*.

Figure 6. Salinity data from sump and experimental culture system during the high inclusion experiment.



As noted in **Table 14**, water quality parameters were acceptable. TAN, Nitrite, and Nitrate values were all well below toxic levels for shrimp, and pH values were within acceptable ranges.

Table 14. Total ammonia nitrogen (mg/kg), nitrite (mg/kg), nitrate (mg/kg), and pH of experimental culture system and sump in the high inclusion experiment. Blank values are from weeks when data was unavailable.

	Sump				Culture tank			
Date	**TAN**	**Nitrite**	**Nitrate**	**pH**	**TAN**	**Nitrite**	**Nitrate**	**pH**
6/6/2012	0.03	-	0.60	8.05	0.02	-	0.40	8.06
6/17/2012	0.14	0.008	-	8.10	0.17	0.067	-	7.84
6/20/2012	0.18	0.064	-	7.80	0.21	0.710	-	7.85
6/27/2012	0.20	0.100	-	7.77	0.18	0.710	-	7.86
7/5/2013	0.00	0.066	33.16	7.79	0.00	0.068	5.53	7.82
7/11/2013	0.21	0.093	6.52	7.75	0.16	0.098	5.20	7.80

3.2 Survival, Growth, and Biomass

3.2.1 Low Inclusion Experiment

Survival was as expected at the Texas AgriLife Research Shrimp Mariculture Project under optimal conditions, verifying that water quality conditions and parameters were optimal for survival. Thus, because survival was high and consistent among treatments (100% in test diets and 93% in the basal diet) biomass values reflected changes in weight. **Table 15** shows survival, growth, and biomass data from the low inclusion experiment.

Table 15. Survival, growth, biomass and weight gain of *L. vannamei* exposed to elevated levels of dietary Fe and Al over 42 days in the low inclusion experiment (initial weight 0.45±0.02 g).

		Supplemental Iron Levels in mg/kg & (%)					
Parameter	**Basal Diet**	**200 (0.02)**	**500 (0.05)**	**1000 (0.10)**	**2000 (0.20)**	**3000 (0.30)**	ANOVA
Survival (%)	93^{a}	100^{b}	100^{b}	100^{b}	100^{b}	100^{b}	P= <0.0001
Weight gain (%)	1434^{d}	1407^{d}	1600^{bcd}	1736^{ab}	1512^{bcd}	1471^{cd}	P= <0.0002
Biomass (g/m^3)	1476^{c}	1668^{bc}	1834^{abc}	1909^{ab}	1696^{bc}	1697^{bc}	P= <0.0080
Weight gain (g/shrimp)	6.39^{e}	6.82^{bcde}	7.40^{abcd}	7.73^{abc}	6.82^{bcde}	6.65^{cde}	P= <0.0001
		Supplemental Aluminum Levels in mg/kg & (%)					
Parameter	**Basal Diet**	**200 (0.02)**	**500 (0.05)**	**1000 (0.10)**	**2000 (0.20)**	**3000 (0.30)**	ANOVA
Survival (%)	93^{a}	100^{b}	100^{b}	100^{b}	100^{b}	100^{b}	P= <0.0001
Weight gain (%)	1434^{d}	1489^{bcd}	1623^{abcd}	1873^{a}	1748^{ab}	1707^{abc}	P= <0.0002
Biomass (g/m^3)	1475^{d}	1720^{bc}	1836^{abc}	2080^{a}	1978^{ab}	1894^{abc}	P= <0.0080
Weight gain (g/shrimp)	6.28^{e}	6.91^{bcde}	7.41^{abcd}	8.46^{a}	7.83^{ab}	7.67^{abc}	P= <0.0001

Percent weight gain and biomass increased from the basal control in significant increments until the 1000 mg/kg inclusion of both Fe and Al. At inclusions above 1000 mg/kg, there was a significant decrease in growth between the 2000 and 3000 mg/kg inclusions. Finally, an estimation of the maximum growth per week based upon a standard growth curve and the linear growth rate was between 2.7 and 2.8 g/week.

3.2.2. High Inclusion Experiment

For the high inclusion experiment, there was no statistical difference in survival. Additionally, because survival was consistent among treatments, biomass values reflected changes in growth. **Table 16** shows survival, growth, and biomass data from the high inclusion experiment.

Table 16. Survival, growth, biomass and weight gain in *L. vannamei* exposed to elevated levels of dietary Al over 42 days for the high inclusion experiment (initial weight 0.9±0.05g). All ANOVA values were $P < 0.0001$.

	Supplemental Iron Levels in % & (ppm)						
Parameter	**Basal (0)**	**1650 (0.17)**	**3260 (0.33)**	**4910 (0.49)**	**6640 (0.66)**	**8290 (0.83)**	**10044 (1.00)**
Survival (%)	93	93	85	88	75	88	90
Weight gain (%)	1705^{a}	1245^{bcd}	1166^{cd}	1119^{cd}	1126^{cd}	1001^{d}	1162^{cd}
Biomass (g/m^3)	2394^{a}	1839^{abcd}	1640^{bcd}	1653^{bcd}	1407^{d}	1490^{cd}	1685^{bcd}
Weight gain (g/shrimp)	14.70^{a}	10.84^{cd}	10.66^{d}	10.38^{de}	10.34^{de}	8.95^{e}	10.24^{de}
	Supplemental Aluminum Levels in % & (ppm)						
Parameter	**Basal (0)**	**670 (0.07)**	**1330 (0.13)**	**2000 (0.20)**	**2702 (0.27)**	**3370 (0.34)**	**4050 (0.41)**
Survival (%)	93	93	98	93	98	95	80
Weight gain (%)	1705^{a}	1367^{bc}	1340^{bc}	1462^{b}	1302^{bc}	1356^{bc}	1165^{cd}
Biomass (g/m^3)	2394^{a}	2053^{ab}	2142^{ab}	2103^{ab}	2048^{abc}	2122^{ab}	1532^{bd}
Weight gain (g/shrimp)	14.70^{a}	12.32^{bc}	12.29^{bc}	12.71^{b}	11.71^{bcd}	12.46^{b}	10.63^{d}

In all diets with supplemental Fe and Al, percent weight gain and biomass decreased significantly with increasing inclusion of either mineral. However, an estimation of the growth per week based upon a standard growth curve and the linear growth rate was between 2.7 and 2.8 g/week.

3.3 Mineral Retention in Tissues

3.3.1 Low Inclusion Experiment

Tissue mineral retention was determined for the basal, 200 mg/kg and 3000 mg/kg inclusions of Fe and Al. **Table 17** shows the amount of Fe and Al (mg/kg) retained in shrimp tissues.

Fe level in the tail muscle (13 mg/kg) for shrimp fed the basal diet with no supplemental Fe was not different from the Fe level (12 mg/kg) in shrimp fed the diet containing 3000 mg/kg supplemental Fe. However, the Fe level in the hepatopancreas (396 mg/kg) of shrimp fed a diet containing 3000 mg/kg supplemental Fe was significantly greater than the Fe level in the hepatopancreas (118 mg/kg) of shrimp fed a diet with only the replete level of Fe (568 mg/kg) in the diet.

The Al levels in the tail muscle (15 mg/kg) and hepatopancreas (45 mg/kg) of shrimp fed a diet containing 3000 mg/kg supplement Al were significantly greater than the Al levels in the tail muscle (3 mg/kg) and hepatopancreas (9 mg/kg) of shrimp fed a diet with the replete level of Al (298 mg/kg).

All Fe and Al levels in the hepatopancreas were greater than the Fe and Al levels in the tail muscle for all diets. The Al levels in the tail muscle of shrimp fed the basal diet and the diet containing 200 mg/kg Al were less than the Fe levels in the tail muscle of shrimp fed the same diets, but similar (15 and 12 mg/kg) in the tail muscle for shrimp fed the diet containing 3000 mg/kg Fe or Al.

Table 17. Mineral retention in body tissues of *L. vannamei* fed diets supplemented with low inclusions of Fe and Al. Values are in mg/kg and are means of three replicates for supplemental diets and five replicates for the basal diet.

	Supplemental Iron Levels in mg/kg & (%)				
Tissue	**Basal (0)**	**200 (0.02)**	**3000 (0.30)**	**ANOVA Pr > F**	**r^2**
Whole Body	30	24	39	0.078	0.304
Tail Muscle	13	11	12	0.888	0.002
Hepatopancreas	118	171	396	0.0001	0.888
	Supplemental Aluminum Levels in mg/kg & (%)				
Tissue	**Basal (0)**	**200 (0.02)**	**3000 (0.30)**	**ANOVA Pr > F**	**r^2**
Whole Body	15	11	19	0.044	0.377
Tail Muscle	3	2	15	0.0003	0.777
Hepatopancreas	9	9	45	0.0001	0.939

3.3.2 High Inclusion Experiment

For the high inclusion experiment, tissue samples were collected and tested for mineral retention in the tail muscle as well as in the combined head and carapace. **Table 18** shows the amount of mineral (mg/kg) retained in shrimp tissue. The Fe level in the tail muscle (18 mg/kg) for shrimp fed the basal diet with no supplemental Fe was significantly lower than the Fe level (53 mg/kg) in shrimp fed diet containing 10,044 mg/kg supplemental Fe.

The Al level in the tail muscle (29 mg/kg) from shrimp fed the diet containing 4048 mg/kg supplement Al was significantly greater than the Al level in the tail muscle (6 mg/kg) of shrimp fed the basal diet with the lowest level of Al (298 mg/kg).

In the combined head and carapace, Fe level (54 mg/kg) for shrimp fed the basal diet with no supplemental Fe was significantly lower than the Fe level (84 mg/kg) in shrimp fed a diet containing 10044 mg/kg supplemental Fe.

The Al levels in the combined head and carapace (43 mg/kg) from shrimp fed the diet containing 4048 mg/kg supplement Al were significantly greater than the Al levels in the tail muscle (17 mg/kg) from shrimp fed the basal diet with the lowest level of Al (298 mg/kg).

Table 18. Mineral retention in body tissues of *L. vannamei* fed diets supplemented with high inclusions of Fe and Al. Values are in ppm and are means of three replicates for supplemental diets and five replicates for the base diet.

	Supplemental Iron Levels in mg/kg & (%)				
Tissue	**Base (0)**	**1667 (0.17)**	**10044 (1.00)**	**ANOVA Pr > F**	**r^2**
Tail Muscle	18	23	53	0.02	0.52
Head + Carapace	54	57	84	0.01	0.51
	Supplemental Aluminum Levels in mg/kg & (%)				
Tissue	**Base (0)**	**672 (0.07)**	**4048 (0.41)**	**ANOVA Pr > F**	**r^2**
Tail Muscle	6	6	29	0.0001	0.98
Head + Carapace	17	19	43	0.0001	0.92

4. DISCUSSION

Throughout both experiments, hydrology and water quality parameters were excellent. This is verified by the high survival and the exceptionally high growth rate of shrimp in both the high and low inclusion experiments. The estimated growth rate of 2.7-2.8 grams per week in the linear growth phase is much higher than the growth rate reported in clear water systems by Forster et al. (2010) (0.73-1.24 g/week) or by Gong et al. (2011) (2.33 g/week).

There was no effect on survival at any level of supplemental Fe or Al. This is somewhat surprising given the high levels of Fe and Al included in the second experiment. Previous studies have reported that high levels of metals such as cadmium (Cd) (0.4 mg/L) are toxic to Pacific white shrimp (Wu et al., 2008). Looking for a dietary requirement, Davis and Lawrence (1992) included Fe up to 80 mg/kg in shrimp diets, and saw no effect on either growth or survival. With the exception of Davis and Lawrence. (1992), there are no reports in the literature concerning the effect of Fe and Al in the diet or in seawater on survival and growth of shrimp. The high percent change of weight indicates that the length of the experiments was sufficient so that any effect the dietary Fe or Al would have had on the shrimp would have been observed during that time.

In the first experiment, growth increased with the increasing inclusion of dietary mineral until the 1000 mg/kg inclusion, and then decreased with increasing levels of Fe and Al. This pattern continued in the second experiment, with levels of dietary mineral

above 1000 mg/kg, growth declined with increasing inclusions of Fe and Al. Weight gain reflected the same pattern.

The weight gain can also be used to estimate growth rate during the linear growth phase. The maximum estimated growth rate for the first and second experiments were 2.7-2.8 grams per week during the linear growth phase. This high estimated linear growth is higher than reported for *L. vannamei* for research experiments and much higher than obtained for commercial production. The rate of 2.7-2.8 grams per week is slightly higher than the average growth rate of 2.3 grams per week at the Port Aransas mariculture facility (Gong et al., 2011).

Surprisingly, few studies have been conducted on the effects of metals as potential dietary toxicants. Powell et al. (2010) exposed sea urchins to dietary copper, demonstrating a similar trend to the low inclusion experiment of this thesis. Sea urchins exposed to low doses of dietary copper showed increased survival and growth, though the trend was not significant ($P = 0.069$). Higher concentrations (114 mg/kg) of copper reduced both growth and survival. In 2006, Shiau and Jiang exposed *Penaeus monodon* to dietary zinc ranging from 7 to 127 mg/kg, and reported a decrease in growth at dietary inclusions of zinc higher than 35 mg/kg, though no effect was seen on survival.

In the low inclusion experiment, shrimp hepatopancreas significantly increased the amount of mineral retained in the tissue when fed diets supplemented with 2000 mg/kg Fe and 3000 mg/kg of Fe or Al. The whole-body samples of the shrimp showed an increase in the retention of aluminum at the 3000 mg/kg inclusion as well. In the high inclusion experiment, both tail muscle and the combined head and carapace retained

significantly more mineral at high inclusions of Fe and Al (10,044 mg/kg and 4048 mg/kg, respectively).

However, the effects of supplemental levels of Fe or Al on shrimp growth and survival have not been reported. Wu and Chen (2005) reported shrimp exposed to Cd and Zn in sea water retained the metals in muscle tissue, gills and hepatopancreas. Using metallothioneins as biomarkers, Wu and Chen (2005) showed that different metals such as Cd and Zn are retained at different rates and in different patterns with a correlation to the concentration and duration of metal exposure in sea water. After exposing *P. monodon* to dietary zinc, Shiau and Jiang (2006) demonstrated an increase in mineral retention in whole body and the hepatopancreas of shrimp that correlated to the increasing level of dietary zinc. In 2008, Wu et al. studied the histopathological effect on shrimp exposed to environmental Cd and Zn and reported that long-term exposure to Cd and Zn caused histological damage to the hepatopancreas of Pacific white shrimp as well as retention of the mineral.

Although no literature was identified regarding the acceptable levels of either Fe or Al in muscle tissue for human consumption, additional research should be performed to evaluate the increase of mineral retention in shrimp muscle tissue as a supplement or as a toxicant to human diets.

Based on this research, assuming an inclusion of 10% of LEA in shrimp diets, 10,000 mg/kg inclusion of Fe and Al in co-products will not have an effect on shrimp growth or survival.

5. CONCLUSIONS

(1.) The water quality and hydrological parameters, as well as the high survival and growth rate seen in shrimp fed the basal diet emphasize the quality of both experiments.

(2.) There was no significant effect on survival for any level of dietary Fe or Al.

(3.) Significant increases in growth occur with the inclusion of dietary Fe or Al up to 1000 mg/kg.

(4.) Significant decreases in growth occur with the increasing inclusion of Fe or Al above 1000 mg/kg.

(5.) No significant effect on the retention of Fe in shrimp tail muscle up was observed up to 1000 mg/kg, but above 1000 mg/kg there was a significant increase in mineral retention.

(6.) At 1000 mg/kg or higher, accumulation of Al in shrimp tail muscle increased significantly.

(7.) Fe and Al were retained at a higher rate in the hepatopancreas than in shrimp tail muscle.

(8.) Dietary Fe and Al levels in LEA co-products up to 10,000 mg/kg is safe in terms of growth and survival of shrimp, assuming an inclusion of up to 10% in diets.

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APPENDIX A

The composition of the Mineral/Vitamin Premix I. All values in mg/kg except for Retinol and Cholecalciferol which are in iu/kg.

Mineral/Vitamin Premix I Ingredient Levels			
Ingredient	**Level**	**Ingredient**	**Level**
Zinc	46000	Riboflavin	11000
Manganese	1100	Pyridoxine	22000
Copper	12000	Niacine	22000
Retinol	600000	Pantothenic Acid	8000
Cholecalciferol	500000	Biotin	200
Tocopherol	40000	Folic Acid	5000
Thiamine	7000	Cyanocobalamine	40

APPENDIX B

The composition of the Mineral/Vitamin Premix II. All values in mg/kg except for Retinol and Cholecalciferol which are in iu/kg.

Mineral/Vitamin Premix II Ingredient Levels			
Ingredient	**Level**	**Ingredient**	**Level**
Zinc	0	Riboflavin	5500
Manganese	5300	Pyridoxine	11000
Copper	0	Niacine	11000
Retinol	1100000	Pantothenic Acid	4000
Cholecalciferol	500000	Biotin	100
Tocopherol	40000	Folic Acid	2500
Thiamine	3500	Cyanocobalamine	20

APPENDIX C

Feed curve based on an FCR of 2.0, a starting weight of 0.43g per shrimp, and a density of seven shrimp per tank. Weight and weight gain are estimated values per shrimp. All values in grams.

Feed Curve for Low Inclusion Experiment

day	weight	gain	feed	feed size	day	weight	gain	feed	feed size
0	0.454	0.089	1.24	18/14	16	3.639	0.331	4.64	12/10
1	0.543	0.099	1.36	14/12	17	3.97	0.343	4.80	12/10
2	0.641	0.109	1.50	14/12	18	4.313	0.354	4.95	12/10
3	0.75	0.119	1.61	14/12	19	4.667	0.364	5.09	12/10
4	0.868	0.129	1.74	14/12	20	5.031	0.373	5.22	12/10
5	0.997	0.125	1.87	14/12	21	5.404	0.382	5.34	12/10
6	1.122	0.176	2.00	14/12	22	5.786	0.39	5.46	12/10
7	1.297	0.195	2.12	14/12	23	6.175	0.397	5.56	12/10
8	1.492	0.213	2.25	14/12	24	6.572	0.403	5.65	12/10
9	1.705	0.23	2.38	14/12	25	6.975	0.409	5.73	12/10
10	1.935	0.247	2.51	14/12	26	7.384	0.414	5.80	12/10
11	2.181	0.263	2.63	14/12	27	7.798	0.418	5.85	12/10
12	2.444	0.278	2.76	14/12	28	8.217	0.422	5.9	12/10
13	2.722	0.292	2.89	14/12	29	8.638	0.425	5.94	12/10
14	3.014	0.306	3.02	12/10	30	9.063	0.427	5.97	12/10
15	3.32	0.319	3.13	12/10					

APPENDIX D

Feed curve based on an FCR of 2.0, a starting weight of 0.90g per shrimp, and a density of five shrimp per tank. Weight and weight gain are estimated values per shrimp.

Feed Curve for High Inclusion Experiment

day	weight (g)	gain (g)	feed (g)	feed size	day	weight (g)	gain (g)	feed (g)	feed size
0	0.967	0.146	1.24	18/14	22	7.455	0.395	3.36	12/10
1	1.114	0.160	1.36	14/12	23	7.850	0.395	3.36	12/10
2	1.273	0.176	1.50	14/12	24	8.245	0.395	3.36	12/10
3	1.450	0.190	1.61	14/12	25	8.640	0.395	3.36	12/10
4	1.640	0.205	1.74	14/12	26	9.035	0.395	3.36	12/10
5	1.845	0.220	1.87	14/12	27	9.430	0.395	3.36	12/10
6	2.064	0.235	2.00	14/12	28	9.825	0.395	3.36	12/10
7	2.299	0.250	2.12	14/12	29	10.220	0.395	3.36	12/10
8	2.549	0.265	2.25	14/12	30	10.615	0.395	3.36	12/10
9	2.814	0.280	2.38	14/12	31	11.010	0.395	3.36	12/10
10	3.094	0.295	2.51	14/12	32	11.405	0.395	3.36	12/10
11	3.389	0.310	2.63	14/12	33	11.800	0.395	3.36	12/10
12	3.699	0.325	2.76	14/12	34	12.195	0.395	3.36	12/10
13	4.024	0.340	2.89	14/12	35	12.590	0.395	3.36	12/10
14	4.364	0.355	3.02	12/10	36	12.985	0.395	3.36	12/10
15	4.719	0.368	3.13	12/10	37	13.380	0.395	3.36	12/10
16	5.087	0.393	3.34	12/10	38	13.775	0.395	3.36	12/10
17	5.480	0.395	3.36	12/10	39	14.170	0.395	3.36	12/10
18	5.875	0.395	3.36	12/10	40	14.565	0.395	3.36	12/10
19	6.270	0.395	3.36	12/10	41	14.960	0.395	3.36	12/10
20	6.665	0.395	3.36	12/10	42	15.355	0.395	3.36	12/10
21	7.060	0.395	3.36	12/10					